

Submission ID #: 69535

Scriptwriter Name: Poornima G

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Title: Development and Standardization of an Ex Vivo Micromethod for Intracellular Quantification of Vincristine in Primary ALL Cells by LC-MS/MS

Authors and Affiliations:

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Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**
3. **Filming location:** Will the filming need to take place in multiple locations? **No**
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 25

Number of Shots: 47 (15 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Videographer' NOTES:

One technical issue we have is with the audio of one of the researchers. There was a very loud, constant noise at the time, and it was the only time of day he could speak. I believe a filter will be needed to clean up this audio a bit (but I believe it's technically straightforward to do).

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

- 1.1. **Adrielli Soares:** Our goal is to measure intracellular vincristine in clinically relevant samples and investigate drug–cell interactions.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~What are the current experimental challenges?~~

- 1.2. **Adrielli Soares/José Yunes:** The current experimental challenges are optimizing incubation time across models. Additionally, model-dependent drug accumulation limits robustness, especially in primary patient samples.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What research gap are you addressing with your protocol?~~

- 1.3. **Adrielli Soares/José Yunes:** Our protocol overcomes limitations of indirect approaches by enabling direct and sensitive intracellular drug measurement in primary leukemia cells.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~What advantage does your protocol offer compared to other techniques?~~

- 1.4. **Adrielli Soares:** Our approach facilitates direct measurement of intracellular vincristine even if cell input is low.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

What questions will future research focus on?

- 1.5. **Adrielli Soares:** In the future, we will investigate whether differences in intracellular drug accumulation are associated with in vitro resistance and we will explore related mechanisms.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Ethics Committee at the Boldrini Children's Center

Protocol

2. Sample Preparation and Drug Incubation

Demonstrator: Adrielli Soares

- 2.1. To begin, thaw the cryopreserved cell sample by gently swirling the vial in a beaker containing 15 milliliters of distilled water at 37 degrees Celsius [1] until the contents are fully thawed [2].
 - 2.1.1. WIDE: Talent holding a vial and gently swirling it inside a beaker filled with water.
 - 2.1.2. Close-up of the vial contents fully thawed.
- 2.2. Transfer the contents of the vial to a 50-milliliter conical tube containing 15 milliliters of sterile 1× PBS using a sterile disposable Pasteur pipette [1]. Place the tube in the centrifuge set to 300 g for 5 minutes at room temperature [2].
 - 2.2.1. Talent pipetting the thawed cell suspension into a 50-milliliter conical tube containing PBS.
 - 2.2.2. Talent placing the conical tube into the centrifuge.
- 2.3. After centrifugation, discard the supernatant by inverting the tube in a single smooth motion [1].
 - 2.3.1. Talent inverting the centrifuged tube to discard the supernatant.
- 2.4. Now, resuspend the cell pellet in 10 milliliters of AIM V (A-I-M-5) medium using a disposable Pasteur pipette [1]. Count viable cells using an automated counter [2] and measure the average cell size [3].
 - 2.4.1. Talent gently pipetting AIM V medium to resuspend the cell pellet.
 - 2.4.2. Talent loading a sample into the automated cell counter.
 - 2.4.3. Display the automated counter interface showing viable cell count results.
- 2.5. Calculate the volume required to obtain 2.5×10^6 viable cells using a simple proportion based on the measured cell concentration [1].
 - 2.5.1. Talent working with a computer and writing calculations in a notebook.

- 2.6. Vortex the vincristine stock solution well before use [1].
 - 2.6.1. Talent vortexing the vincristine stock solution.
- 2.7. Add AIM V medium to a 2 milliliter microtube [1], followed by 2.5×10^6 cells, and then add the required volume of vincristine solution [2]. Vortex briefly [3] and spin the tube for 5 seconds to collect liquid from the cap [4].
 - 2.7.1. Talent pipetting AIM V medium into a microtube.
 - 2.7.2. Talent adding the calculated volume of cells to the microtube.
 - 2.7.3. Talent briefly vortexing the microtube.
 - 2.7.4. Talent placing the microtube into a centrifuge for a brief spin.
- 2.8. Next, incubate the open microtubes at 37 degrees Celsius with 5 percent carbon dioxide for 3 hours [1].
 - 2.8.1. Talent placing open microtubes into the incubator.
- 2.9. After incubation, close the tube lids [1] and centrifuge the samples at 300 g for 5 minutes at room temperature [2].
 - 2.9.1. Talent closing the lids of the microtubes.
 - 2.9.2. Talent placing the microtubes into the centrifuge.
- 2.10. Carefully discard the supernatant using a pipette [1], then add 1 milliliter of PBS at room temperature to the tube [2].
 - 2.10.1. Talent aspirating and discarding the supernatant with a pipette.
 - 2.10.2. Talent adding PBS to the tube.
- 2.11. After repeating the centrifugation and wash steps, carefully remove all remaining supernatant using a pipette [1], resuspend the pellet in 100 microliters of PBS [2], and vortex until the pellet is fully resuspended [3].
 - 2.11.1. Talent removing residual supernatant with a pipette.
 - 2.11.2. Talent adding PBS to the pellet.
 - 2.11.3. Talent vortexing the tube until the pellet is fully resuspended.

2.12. Now, add 400 microliters of cold methanol containing 0.1 micromolar vinblastine at minus 20 degrees Celsius directly to the sample while avoiding contact with the tube walls [1].

2.12.1. Talent carefully pipetting cold methanol with vinblastine directly into the sample.

2.13. Store the samples at minus 80 degrees Celsius overnight [1].

2.13.1. Talent placing labeled samples into a minus 80-degrees Celsius freezer.

3. Sample Processing for LC-MS/MS

3.1. Pre-cool the centrifuge to 4 degrees Celsius before removing the samples from the minus 80 degrees Celsius freezer [1].

3.1.1. Talent setting the centrifuge temperature to 4 degrees Celsius and confirming the setting on the control panel.

3.2. Immediately after removing the samples from the freezer [1], centrifuge them at 13,000 *g* for 20 minutes at 4 degrees Celsius [2].

3.2.1. Talent removing frozen samples from the freezer.

3.2.2. Talent placing the samples in a centrifuge.

3.3. Then, transfer 300 microliters of the supernatant to a 1.5-milliliter microtube using a pipette [1].

3.3.1. Talent carefully pipetting the supernatant into a labeled 1.5 milliliter microtube.

3.4. Dry the samples completely by placing them in a vacuum concentrator set to 45 degrees Celsius for 1 hour and 40 minutes [1].

3.4.1. Talent placing open microtubes into the vacuum concentrator.

3.5. After drying, resuspend each pellet in 400 microliters of water containing 0.1 percent formic acid using a pipette [1]. Vortex gently until the pellet is fully dissolved [2].

- 3.5.1. Talent adding the prepared water and formic acid solution to each tube.
- 3.5.2. Talent gently vortexing the tubes to dissolve the pellets.

3.6. Now, centrifuge the samples again at 13,000 *g* for 20 minutes at 4 degrees Celsius [1].

- 3.6.1. Talent placing the resuspended samples into the centrifuge.

3.7. Transfer 300 microliters of the supernatant to a 2-milliliter screw-top vial for liquid chromatography–tandem mass spectrometry analysis [1].

- 3.7.1. Talent pipetting the supernatant into labeled 2 milliliter screw-top vials.

4. LC-MS/MS Data Acquisition and Analysis

4.1. On the software home screen, open **Sample List [1]** and create a new list by entering the sample names, injection volumes, vial positions, and the method to be used [2].

- 4.1.1. SCREEN: 69535_screenshot_1.mp4 00:00-00:08.

- 4.1.2. SCREEN: 69535_screenshot_1.mp4 00:09-00:15 and 00:32-00:36.

4.2. Place a vial containing ultrapure water into the autosampler tray [1] and designate this vial as a blank in the Sample List [2]. Run the blank between every set of triplicate injections to avoid carryover [3].

- 4.2.1. Talent placing a vial of ultrapure water into an open position in the autosampler tray.

- 4.2.2. SCREEN: 69535_screenshot_2.mp4. 00:00-00:05

- 4.2.3. SCREEN: 69535_screenshot_2.mp4. 00:06-00:08

4.3. Select the desired rows in the Sample List [1] and begin data acquisition by clicking **Run** and confirming with **OK [2]**.

- 4.3.1. SCREEN: 69535_screenshot_3.mp4. 00:00-00:02.

- 4.3.2. SCREEN: 69535_screenshot_3.mp4. 00:03-00:13.

4.4. After the run completes, select a sample from the results [1] and view the chromatograms by clicking **Chromatogram** in the top menu bar [2].

4.4.1. SCREEN: 69535_screenshot_4.mp4. 00:00-00:05.

4.4.2. SCREEN: 69535_screenshot_4.mp4. 00:06-00:13.

4.5. Finally, on the home screen, click **TargetLynx** (*target-lynx*) [1], select the files that just ran [2], and click Process. Select the vincristine method and click **OK** to generate output tables [3].

4.5.1. SCREEN: 69535_screenshot_5.mp4. 00:00-00:02.

4.5.2. SCREEN: 69535_screenshot_5.mp4. 00:03-00:04.

4.5.3. SCREEN: 69535_screenshot_5.mp4. 00:05-00:18.

Results

5. Results

5.1. Chromatographic analysis of primary acute lymphoblastic leukemia patient-derived xenograft cells incubated with 1 micromolar vincristine for 3 hours consistently showed a symmetrical and well-resolved VCR peak at 2.85 minutes, confirming appropriate retention and minimal matrix interference [1].

5.1.1. LAB MEDIA: Figure 1. *Video editor: Highlight the VCR peaks at 2.85 min in each chromatogram.*

5.2. Calibration curves for vincristine generated in AIM V media showed strong linearity across concentrations from 0.001 to 10 micromolar [1]. Similarly, when 2×10^6 RS4;11 (*R-S-4-eleven*) cells were added, a linear response was observed from 0.01 to 1 micromolar [2].

5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the plot labelled "A" .*

5.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight the plot labelled "B".*

5.3. Time-course analysis of vincristine accumulation revealed that RS4;11 cells exhibited a plateau in the VCR/VINB (*vincristine by vinblastine*) ratio between 2 and 3 hours [1], with a modest increase at 4 and 5 hours [2], while primary PDX cells peaked at 2 hours [3] and declined thereafter, supporting the selection of 3 hours as a balanced incubation time [4].

5.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight bars at 2 h and 3 h in panel B (RS4;11 cells).*

5.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight the bars 4 h and 5 h in panel B.*

5.3.3. LAB MEDIA: Figure 3. *Video editor: Highlight the bar at 2 h in panel A (PDX cells).*

5.3.4. LAB MEDIA: Figure 3. *Video editor: Show the bars from 2 h to 5 h in panel A.*

5.4. Normalized vincristine accumulation varied across six PDX patient samples, and a positive correlation was observed between unnormalized VCR/VINB ratios and average cell volume [1].

5.4.1. LAB MEDIA: Figure 4.

5.5. Fragmentation analysis confirmed that the base peak of vincristine corresponded to the precursor ion at mass-to-charge ratio 413.38 [1], which generated a quantification ion

at 392.47 [2] and a confirmation ion at 383.38 under 40 volts cone energy and 16 volts collision energy [3].

5.5.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the tall peak labelled m/z 413.38 in panel A.*

5.5.2. LAB MEDIA: Figure 5A. *Video editor: Highlight the peak labelled m/z 392.47 in panel A.*

5.5.3. LAB MEDIA: Figure 5A. *Video editor: Highlight the peak labelled m/z 383.38 in panel A.*

- Cryopreserved

Pronunciation link: <https://www.merriam-webster.com/dictionary/cryopreserved>

IPA: /ˈkraɪoʊprɪˈzɜːvd/

Phonetic Spelling: kry-oh-prih-zurv

- Conical

Pronunciation link: <https://www.merriam-webster.com/dictionary/conical>

IPA: /ˈkɑːnɪkəl/

Phonetic Spelling: kah-nih-kuhl

- Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /ˈsentrəˌfjuːdʒ/

Phonetic Spelling: sen-truh-fyooj

- Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

IPA: /ˌsuːpərˈnɛtənt/

Phonetic Spelling: soo-per-nay-tuhnt

- Resuspend

Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>

IPA: /ˌriːsəˈspend/

Phonetic Spelling: ree-suh-spend

- Pellet

Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>

IPA: /ˈpɛlɪt/

Phonetic Spelling: peh-liht

- Vincristine

Pronunciation link: <https://www.merriam-webster.com/dictionary/vincristine>

IPA: /vɪnˈkrɪstɪn/

Phonetic Spelling: vin·kris·teen

- Vinblastine

Pronunciation link: <https://www.merriam-webster.com/dictionary/vinblastine>

IPA: /vɪnˈblæstɪn/

Phonetic Spelling: vin·blas·teen

- Micromolar

Pronunciation link: <https://www.merriam-webster.com/dictionary/micromolar>

IPA: /ˌmaɪkroʊˈmoʊlər/

Phonetic Spelling: my·kroh·moh·ler

- Methanol

Pronunciation link: <https://www.merriam-webster.com/dictionary/methanol>

IPA: /ˈmɛθəˌnɔːl/

Phonetic Spelling: meth·uh·nawl

- Xenograft

Pronunciation link: <https://www.merriam-webster.com/dictionary/xenograft>

IPA: /ˈzɛnəˌgræft/

Phonetic Spelling: zeh·nuh·graft

- Lymphoblastic

Pronunciation link: <https://www.merriam-webster.com/dictionary/lymphoblastic>

IPA: /ˌlɪmfoʊˈblæstɪk/

Phonetic Spelling: lim·foh·blas·tik

- Chromatographic

Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatographic>

IPA: /ˌkroʊməˈtægrəfɪk/

Phonetic Spelling: kroh·muh·tag·ruh·fik

- Chromatogram

Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatogram>

IPA: /kroʊˈmætəˌgræm/

Phonetic Spelling: kroh·mat·uh·gram

- Fragmentation

Pronunciation link: <https://www.merriam-webster.com/dictionary/fragmentation>

IPA: /ˌfrægmənˈteɪʃən/

Phonetic Spelling: frag·men·tay·shuhn

- Precursor

Pronunciation link: <https://www.merriam-webster.com/dictionary/precursor>

IPA: /prɪˈkɜːsər/

Phonetic Spelling: prih·kur·ser

- Autosampler

Pronunciation link: <https://www.merriam-webster.com/dictionary/autosampler>

IPA: /ˌɔːtoʊˈsæmplər/

Phonetic Spelling: aw·toh·sam·pler

- Ultrapure

Pronunciation link: <https://www.merriam-webster.com/dictionary/ultrapure>

IPA: /ˌʌltrəˈpjʊr/

Phonetic Spelling: uhl·truh·pyur

- Formic

Pronunciation link: <https://www.merriam-webster.com/dictionary/formic>

IPA: /ˈfɔːrmɪk/

Phonetic Spelling: for·mik

- Vortex

Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>

IPA: /ˈvɔːrtɛks/

Phonetic Spelling: vor·teks